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Biochemistry

Identification of the polypeptide chains in *Torpedo californica* electroplax membranes that interact with a local anesthetic analog

(acetylcholine receptor/photoaffinity labeling/procaine amide azide/histrionicotoxin binding)

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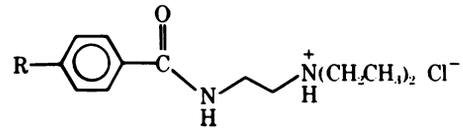
ABSTRACT **Procaine amide azide, a derivative of the local anesthetic procaine amide, was prepared and its interactions with acetylcholine receptor-rich membrane fragments from *Torpedo californica* electroplax were studied. Procaine amide azide was radiolabeled by a method that may be of general use for the preparation of other radioactive tertiary amines. When low concentrations of ^{3}H-labeled procaine amide azide were photolyzed in the presence of receptor-rich membrane preparations, a simple pattern of incorporated radioactivity was seen after electrophoresis on sodium dodecyl sulfate/polyacrylamide gels. Only two major labeled bands were seen, corresponding to apparent M_r of about 43,000 and 90,000. When the length of the gels was increased, the labeled band of lower M_r was resolved into two labeled proteins, one major and one minor, with apparent M_r of 43,000 and 40,000, respectively. The radioactivity incorporated into the protein of M_r 40,000 could be attributed to interaction of [^{3}H]procaine amide azide with cholinergic ligand-binding sites, whereas labeling of the polypeptide of M_r 43,000 appears to represent interaction of the photolabile derivative with another class of sites. The labeling component of 90,000 M_r could be removed by preparation of membrane fragments in iodoacetamide-containing buffer and therefore appeared unrelated to the acetylcholine receptor.**

Membrane fragments with high concentrations of nicotinic acetylcholine receptor (AcChoR) have been isolated from the electric tissue of several electric rays (1–3) and the electric eel *Electrophorus* (4). The *in vitro* characterization of these systems has focused largely on the ligand-binding properties of the AcChoR recognition site. These ligands may be classified as (*i*) acetylcholine (AcCho) and compounds of similar pharmacological action (agonists), (*ii*) compounds that inhibit depolarization (antagonists; e.g., *d*-tubocurare), and (*iii*) α-bungarotoxin (α-BuTx) and related snake neurotoxins that specifically and irreversibly block AcCho response *in vivo* (5, 6) by binding to the AcChoR. The effects *in vivo* of several compounds whose primary site of interaction is distinct from the AcCho recognition site suggest that they may be useful probes for the membrane component or components related to ion translocation. One such compound is the alkaloid histrionicotoxin (HTX), first isolated by Daly *et al.* (7). *In vitro* studies have shown that membrane fragments from *Torpedo californica* bind ^{3}H-labeled H_{12}-HTX in a specific manner with a K_d of 0.3–0.5 μM and a stoichiometry of 1 HTX site to 4 α-BuTx sites (8) in addition to a large nonspecific binding component. Caeruleotoxin was considered as another candidate for a specific probe of the "ionic conductance modulator" (9). However, postsynaptic inhibition by this polypeptide has been attributed to its phospholipase activity (10). Local anesthetics comprise another class of potentially useful compounds for study of mechanisms related to ion channel function.

At the neuromuscular junction, local anesthetics alter the normal time course of end-plate currents induced by AcCho; whereas normal end-plate currents decay with a single exponential time course, the decay becomes multiphasic in the presence of procaine and other local anesthetics (11–15). Recent studies (16–18) have led to proposals that this action might be explained by binding of a local anesthetic to the open channel (i.e., conducting) form of the receptor, resulting in a decreased ionic conductance. These compounds, then, might be used as probes for the structure(s) involved in translocation of ions across the postsynaptic membrane and its relationship to (or identity with) the AcChoR.

The interactions of various local anesthetics with receptor-rich membrane fragments have been studied by means of inhibition of α-toxin binding (19, 20) and by extrinsic fluorescent probes (21–23). In the studies reported here, we prepared an azido analog of the local anesthetic procaine amide (**I**); namely, procaine amide azide (PAA) (**II**). This compound was chosen over the procaine derivative (24) because the amide bond is less susceptible to hydrolysis than is procaine's ester linkage. We have previously shown that the membranes contain polypeptides of M_r 40,000, 50,000, 60,000, and 65,000 (2, 3) in addition to other major species of M_r ≈43,000 and 90,000 (25). Photolysis of [^{3}H]**II** in the presence of AcChoR-rich membrane fragments was coupled with sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis to demonstrate which polypeptides of the membrane fragment preparation were involved in local anesthetic binding.



(I) R = NH_2; (II) R = N_3

MATERIALS AND METHODS

Preparation of AcChoR Membrane Fragments. *T. californica* were obtained locally. After they were killed, the electric organs were excised, frozen in liquid nitrogen, and then stored at −90°C until use. AcChoR-enriched membrane fragments were prepared as described (2, 3) or by a modification of these methods. In the latter case, centrifugation on a discontinuous sucrose gradient in a Beckman VTi-50 reorienting tube rotor replaced the zonal centrifugation step. In this way the gradient centrifugation time was reduced from overnight to about 1 hr. Specific activities were comparable to those obtained by zonal centrifugation (unpublished data). When

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Abbreviations: AcCho, acetylcholine; AcChoR, acetylcholine receptor; α-BuTx, α-bungarotoxin; PAA, procaine amide azide; Carb, carbamylcholine; HTX, histrionicotoxin; $NaDodSO_4$, sodium dodecyl sulfate.

membrane fragments were treated with iodoacetamide, it was added during homogenization to a concentration of 1 mg/ml of homogenate. The buffer in subsequent steps of the purification did not contain iodoacetamide. The concentration of α-BuTx binding sites was measured by the method of Schmidt and Raftery (26); protein was measured by the method of Lowry *et al.* (27).

Synthesis and Characterization of PAA. Unlabeled PAA was synthesized from procaine amide·hydrochloride (Pfaltz and Bauer, Inc., Stamford, CT) by preparation of the corresponding diazonium salt followed by its reaction with sodium azide. The identity of the product was confirmed by elemental analysis. Calculated for $C_{13}H_{20}N_5ClO$: C, 52.4; H, 6.77; N, 23.5; Cl, 11.9. Found: C, 52.6; H, 6.8; N, 23.4; Cl, 12.2. The synthesis of [^{3}H]PAA is outlined in Fig. 1. The interactions of PAA with membrane-bound AcChoR were characterized in several ways. The apparent inhibition constant of PAA towards α-BuTx binding was determined by inhibition of the initial rate of ^{125}I-labeled α-BuTx binding caused by varying concentrations of the ligand. For details, see the legend of Fig. 2*A*. The rate of the transition of the receptor from low to high affinity caused by 1 μM carbamylcholine (Carb) in the presence and absence of PAA was measured by the method of Lee *et al.* (30). The effects of PAA on the fluorescence of membrane-bound ethidium bromide (Calbiochem) were measured as described (22).

Labeling and Polyacrylamide Gel Electrophoresis. Membrane fragments were incubated with the desired concentration of [^{3}H]PAA in the dark for 20–30 min at 0°C. The solutions were irradiated for 2 min in a quartz cuvette (with constant stirring) by using a UVSL·25 lamp (Ultraviolet Products, San Gabriel, CA) on the short-wavelength setting. The membranes were centrifuged for 15 min in an Eppendorf model 3200 centrifuge, and the supernatant was discarded. The pellet was resuspended in 1 ml of *Torpedo* Ringer's solution and the membranes were repelleted as above. After the supernatant was discarded, the pellets were dissolved in sample buffer [10% (wt/vol) glycerol/3% (wt/vol) $NaDodSO_4$/100 mM dithiothreitol/62.5 mM Tris·HCl, pH 6.8] and denatured by heating at 100°C for 2 min. $NaDodSO_4$/polyacrylamide gel electrophoresis was carried out in the system of Laemmli (31). Gels were either 1 × 10 cm or, when it was desired to distinguish between label on the 40,000 and 43,000 M_r bands, 1 × 20 cm. In both cases the separating gels were 8.75% (wt/vol) in acrylamide.

After electrophoresis the gels were either sliced immediately or after staining in 10% HOAc/25% MeOH/0.05% Coomassie Brilliant Blue and destaining in the same solution without the dye. Gels were sliced with a Hoeffer Scientific Instruments SL-280 gel slicer. Each slice was placed in a scintillation vial with 0.5 ml of 30% H_2O_2; the vials were tightly capped and heated at 70°C for 5 hr. After cooling and addition of scintillation fluid, the radioactivity in the vials was determined by using the tritium channel of a Packard 3375 liquid scintillation counter. For the 20-cm gels, radioactivity was measured in only that portion of the gel containing the 40,000–90,000 M_r bands because initial experiments showed no significant incorporation of radioactivity outside of this M_r region.

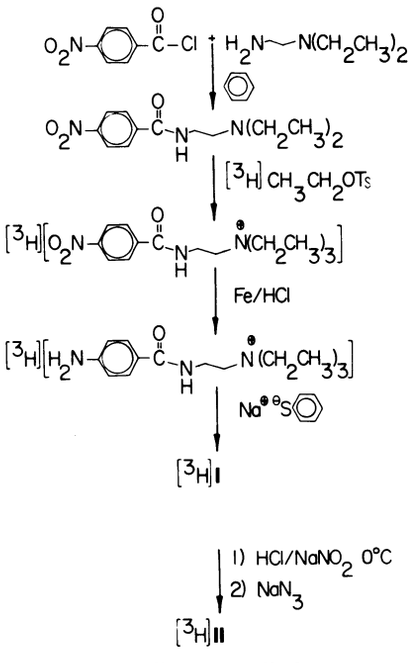

FIG. 1. Synthesis of [^{3}H]PAA. [^{3}H]Ethyl tosylate ([^{3}H]-CH_3CH_2OTs) was prepared from [1-[^{3}H](*N*)]ethanol (93.6 Ci/mol, New England Nuclear) and tosyl chloride in dry pyridine (28). Sodium thiophenoxide was prepared by a modification of the method of Jenden and Hanin (29). In the dequaternization step, there was a 2/3rd probability that a radioactive ethyl group would not be removed. The expected specific activity of the final product was therefore 2/3rd that of the ethyl tosylate or 62.4 Ci/mol. After purification on a silica gel column, the concentration of the [^{3}H]PAA stock solution was calculated from its absorbance at 270 nm, with unlabeled PAA as standard. The specific activity was determined by liquid scintillation counting of aliquots of the stock solution and found to be 54 Ci/mol ± ≈10%, in agreement with the predicted value.

RESULTS

PAA only weakly inhibited ^{125}I-labeled α-BuTx binding to membrane-bound AcChoR but was slightly more effective than its parent compound in this respect (Fig. 2*A*). Titration of membrane fragments with PAA by using the fluorescent probe ethidium (Fig. 2*B*) showed the same qualitative effects as seen with the local anesthetic procaine (22). When the data were corrected for nonspecific (i.e., not displaceable by α-BuTx) effects, a fluorescence increase was observed in the micromolar range, whereas addition of millimolar concentrations of PAA caused a decrease in the signal (Fig. 2*B*).

Since there was evidence for PAA binding to some site with an affinity higher than that measured by ^{125}I-labeled α-BuTx inhibition (i.e., the fluorescence enhancement, Fig. 2*B*) and since local anesthetics have been reported to increase the rate of the Carb-induced transition in receptor affinity at concentrations below those that directly inhibit α-BuTx binding (20, 32), the effects of PAA on the rate of this process were examined. Up to concentrations of 50 μM PAA, the rate of the transition of the receptor from low to high affinity caused by 1 μM Carb (30) was only slightly changed. The half-time of 97 ± 23 sec (SD) for this process in the presence of 1 μM Carb alone was reduced to only 71 ± 16 sec at the highest concentration of PAA tested, a 1.4 ± 0.4-fold decrease.

Fig. 3*A* shows a $NaDodSO_4$ gel electrophoresis profile of membrane fragments that were photolabeled with [^{3}H]PAA. Only two labeled bands were seen. Although the relative mobility of the faster migrating band corresponded to a M_r of about 42,000–44,000, the limited resolution of the gels in this region made it impossible to determine if the M_r 40,000 receptor band also contained bound label. The amount of radioactivity incorporated in the M_r 90,000 band varied from preparation to preparation, as did the intensity of its Coomassie Brilliant Blue staining. When membrane fragments homogenized in the presence of iodoacetamide were labeled under the same conditions, almost no radioactivity was incorporated into the M_r 90,000 band (Fig. 3*B*). The decrease in the intensity of

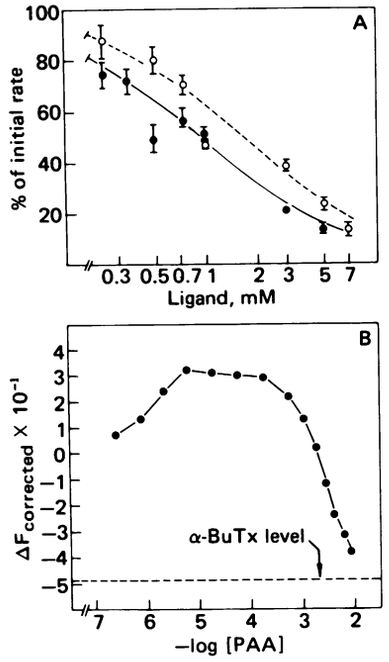

FIG. 2. (*A*) Inhibition of initial rate of ^{125}I-labeled α-BuTx binding as a function of ligand concentration. Membrane fragments 63 nM in toxin-binding sites were incubated with the indicated concentration of ligand for 20 min and 470 nM ^{125}I-labeled α-BuTx was added to start the reaction. The initial rates, as determined from semilogarithmic plots of the data, were fit to a linearized form of the empirical relation $k_{obs} = k/(1 + L/K_i)$, in which k_{obs} was the initial rate in the presence of ligand concentration L, k the rate in the absence of ligand, and K_i the apparent inhibition constant. The line represents calculated K_i values of 0.95 ± 0.06 mM for PAA (●) and 1.7 ± 0.1 (SD) mM for procaine amide (○). (*B*) Effects of PAA on fluorescence of membrane-bound ethidium. Concentrations of α-BuTx binding sites and of ethidium were both 1 μM. $\Delta F_{corrected}$ is the change in ethidium fluorescence from the initial level at [PAA] = 0 for membrane fragments alone minus the corresponding change for membrane fragments that were incubated with an excess of α-BuTx.

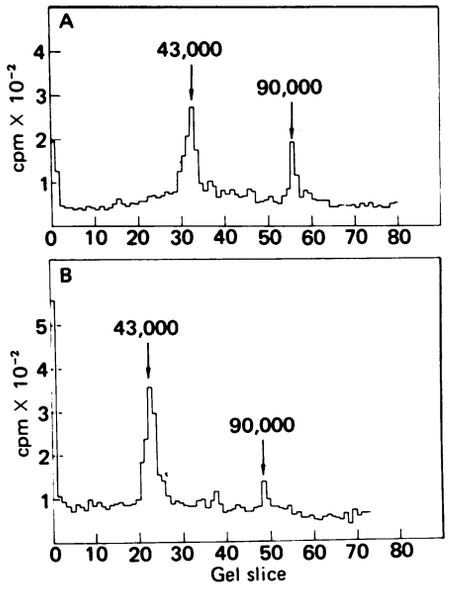

FIG. 3. Polyacrylamide gel electrophoresis profiles of AcChoR-enriched membrane fragments labeled with [^{3}H]PAA. (*A*) Solution 0.5 μM in ^{125}I-labeled α-BuTx binding sites and 5 μM in [^{3}H]PAA was treated as described in *Materials and Methods*. A total of 0.75 nmol of ^{125}I-labeled α-BuTx binding sites was placed on the gel. (*B*) Concentrations were the same as *A* except that the membrane fragments were homogenized in the presence of iodoacetamide. One nanomole of ^{125}I-labeled α-BuTx sites was placed on the gel. Gel slice 1 is the position of the tracking dye; the last slice is the top of the gel.

the Coomassie Blue staining was less dramatic. Two possibilities could explain the changed labeling pattern in iodoacetamide-treated membrane fragments: (*i*) the component of M_r 90,000 labeled by PAA was lost during the purification process, presumably because artifactual disulfide bonds could not form or (*ii*) iodoacetamide altered the binding properties of the membrane fragments for PAA and this change was reflected in the labeling of the M_r 90,000 band. Because this band *was* labeled when membranes were treated with iodoacetamide *after* preparation (not shown), the second possibility could be ruled out. In accord with the conclusion that iodoacetamide treatment did not perturb the interactions of PAA with membrane fragments, the ethidium fluorescence signal, when membranes were titrated with PAA, was unchanged. Both the increased fluorescence at low concentrations and the decrease in signal at higher ones were observed.

When membranes were run on gels 20 cm long, the separation between the 40,000 and 43,000 M_r bands was increased. As Fig. 4 shows, both bands were labeled, with the majority of the radioactivity in the higher M_r band. Photolysis of membrane fragment solutions containing both [^{3}H]PAA and 2–5 μM Carb resulted in decreased incorporation of radioactivity into the 40,000 M_r but not the 43,000 M_r band (or the residual 90,000 M_r label, not shown). The M_r 40,000 subunit of *T. californica* contains sites for binding of α-toxins (33, 34) and cholinergic ligands, such as the antagonists 4-(*N*-maleimido)-benzyltrimethylammonium iodide (35) and bis-3-aminopyridinium-1-azide (25) and the agonist bromacetylcholine (unpublished data). The label incorporated into the polypeptide of M_r 40,000 was therefore attributed to PAA binding at these site(s). The small amount of bound radioactivity was probably a reflection of the low affinity (determined by inhibition of ^{125}I-labeled α-BuTx binding) of these site(s) for the anesthetic analog.

DISCUSSION

The postsynaptic membrane responds to cholinergic agonists *in vivo* by a transient increase in cation permeability, and this response may be directly inhibited by compounds that block the binding of agonists. The snake neurotoxin α-BuTx, whose blockage is irreversible (5, 6), is especially useful in this respect. The response to agonists may also be indirectly blocked by conversion of the AcChoR–ion translocation complex to an inactive conformation (desensitization) or by perturbation of the structure(s) involved in ion translocation or of its coupling to the AcChoR. Membrane fragments from *Torpedo* electroplax exhibit a number of properties *in vitro* consistent with *in vivo* observations at the neuromuscular junction and therefore constitute a good system for the study of postsynaptic function: (*i*) these membranes bind agonists and antagonists, and this binding is irreversibly blocked upon addition of α-BuTx; (*ii*) Carb induces increased ion flux from *Torpedo* membrane fragments *in vitro*, as measured by release of $^{22}Na^+$, and this release is inhibited by both α-BuTx (36–38) and by HTX, a noncompetitive inhibitor (unpublished data); and (*iii*) in addition, exposure to cholinergic agonists causes a reversible increase in the AcChoR's ligand affinity (20, 30, 39–41), a process that may be an *in vitro* correlate to the *in vivo* phenomenon of desensitization. Tertiary amine local anesthetics have been reported to affect the rate of both the *in vivo* process of densi-

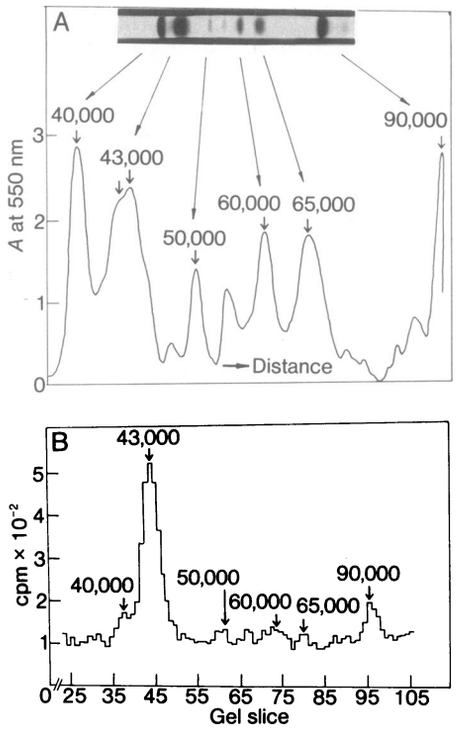

FIG. 4. Improved separation of membrane components on 1 × 20 cm $NaDodSO_4$/polyacrylamide gels. (*A*) Scan of Coomassie Brilliant Blue staining intensity. The central portion of a 1 × 20 cm gel was scanned with a Gilford Linear transport accessory with a specially made cuvette and holder. Note that the region around M_r 43,000 contained several staining bands that were not well resolved. The relative amounts of each polypeptide present could not be quantitated due to the very high absorbance and the fact that the stain did not always penetrate to the center of the gels because of their large diameter. (*B*) Radioactivity profile. A total of 2 nmol of ^{125}I-labeled α-BuTx sites was placed on the gel. The labeling conditions were identical to those in Fig. 3*B*. The major amount of radioactivity was incorporated at a position corresponding to a M_r of 43,000 with minor incorporation at M_r 40,000 and 90,000. Each gel slice was 1.1 mm long, with a total of 152 slices from the dye front to the top of the gel.

tization (42, 43) and the *in vitro* affinity change (refs. 20 and 32; unpublished data). *In vivo*, local anesthetics also interfere with the mechanism of ion translocation (11–18).

Since incorporation of [^{3}H]PAA into the protein of M_r 40,000 was prevented by the presence of Carb, the labeling seen on this polypeptide could be interpreted as being due to PAA bound at cholinergic ligand-binding sites. The identity of the labeled band of M_r 43,000 was less clear. Sobel *et al.* (44) have recently reported isolation of a particulate protein fraction of this molecular weight from *T. marmorata* membrane fragments. These authors observed that the fluorescence of quinacrine associated with this 43,000 M_r protein was decreased upon addition of HTX in a manner similar to the effects of HTX on quinacrine fluorescence in preparations of intact membrane fragments. It was not possible, however, to equate the protein of Sobel *et al.* with the band of M_r 43,000 labeled by [^{3}H]PAA because the Coomassie Blue staining intensity of high resolution (20 cm) gels of *T. californica* membrane fragments indicated the presence of more than one protein with approximately this molecular weight (Fig. 4). Because of limitations in the resolution of these gels, we could not unambiguously determine which partially resolved component was labeled by [^{3}H]PAA. In addition, preliminary results showed that the presence of micromolar concentration of HTX did not decrease the amount of radioactivity incorporated in the 43,000 M_r band when *T. californica* membranes were photolyzed in the presence of [^{3}H]PAA. Conversely, unlabeled PAA displaced membrane-bound ^{3}H-labeled H_{12}-HTX with an apparent K_i of 500 μM (unpublished data), a concentration 2 orders of magnitude higher then that necessary to label membrane fragments with [^{3}H]PAA and, as shown in Fig. 2*B*, well above those concentrations that enhance the fluorescence of membrane-bound ethidium bromide. Thus, although HTX and local anesthetics show similar actions both *in vivo* and *in vitro*, they may bind to separate sites on the same or on different polypeptide chains.

The amount of radioactivity incorporated into the protein of M_r 90,000 varied from one preparation to another. The possibility that this band was due to disulfide bonds between smaller labeled bands was ruled out because dithiothreitol was included in the sample buffer for all $NaDodSO_4$/polyacrylamide gel electrophoresis experiments. The replacement of dithiothreitol with mercaptoethanol also had no effect. Because the 90,000 M_r labeling band was easily separable from membrane fragments by homogenization in the presence of iodoacetamide, it was tentatively assumed to be unrelated to the AcChoR–ion translocation complex. Although we could not rule out that the 90,000 M_r protein might play a role in ion translocation, it is of interest that iodoacetamide treatment did not alter any measured properties *in vitro* of the membrane fragments, except for the [^{3}H]PAA labeling pattern. The association rate constant for α-BuTx binding was unchanged; the membranes underwent the change from low to high affinity caused by Carb and bound HTX in a specific manner (unpublished data). In addition, iodoacetamide-treated membranes still showed agonist-mediated cation flux (unpublished data). Therefore, the assumption that the 90,000 M_r protein was not directly related to the AcChoR complex seemed justified.

Although the data presented here have demonstrated that the local anesthetic analog PAA binds to several components in *T. californica* membrane fragments, the roles these components play in *in vivo* or *in vitro* processes, such as channel blockage of desensitization, are not understood.

The precise mechanisms by which the local anesthetic effects of procaine or procainamide are exerted are not yet resolved. With respect to the possible *in vitro* correlate of densensitization, namely, the conversion of the membrane-bound receptor from a state(s) of low to high affinity by Carb, PAA decreased the half-time of this transition by a factor of only 1.4 ± 0.4 at a concentration 10 times above that used in the labeling experiments. The precision of our assay did not allow us to decide if this represented a significant change in the rate of this transition; however, this finding was not at variance with observations for other local anesthetics such as procaine, since we have observed that the effects on this rate differ both qualitatively and quantitatively for different anesthetics (unpublished observations). Therefore, little or no effect on the rate of this receptor transition at a given concentration of local anesthetic could indicate that either little or no local anesthetic was bound to the site(s), affecting the rate of conversion, or the compound was bound to the site(s), but did not affect the rate to an appreciable extent. Compounds such as some local anesthetics (20, 32), detergents (32, 45), caeruleotoxin (9, 10), and, in some tissues (46) but not others (8), HTX affect the rate of transition of receptor from a state of low to high affinity. However, despite the variety of effects observed for these compounds, the mechanisms by which they exert the effect are poorly understood.

At the present time there are few specific probes useful for identification of the site(s) to which compounds such as HTX or local anesthetics bind to the postsynaptic membrane and

exert their physiological effects. PAA holds promise for elucidation of some of the mechanisms involved.

Note Added in Proof. After submission of this manuscript we found that Levy *et al.* (47) have used a similar approach by using *p*-azidobenzoyldiethylaminoethanol in studies of hepatocyte plasma membranes.

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